

ANALYTICAL INVESTIGATIONS

IN

THE DAIRY FIELD

av

Hans Jönsson

Rund 1977



**DEPARTMENT
OF
TECHNICAL ANALYTICAL
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AVHANDLING FÖR TEKNISK DOKTORSEXAMEN

Avhandlingen kommer att försvaras vid en offentlig disputation
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INTRODUCTION

Milk and products based on milk are some of the most important foods in human nutrition. The gross chemical composition of raw milk is tabled below.

TABLE 1. Important constituents of raw milk¹

Protein	3.1 - 3.7%
Fat	3.5 - 4.6%
Lactose	4.5 - 5.1%
Citric acid	0.15-0.22%
Minerals	
Macro	Na, K, Ca, Mg, P, Cl
Micro	Zn, Cu, Fe, Mn
Vitamins	
Fat soluble	A, D, E
Water soluble	B ₁ , B ₂ , B ₁₂
Total solids	12.0-13.7%
Ashes	0.67-0.78%

By processing, *e.g.* separation, ultrafiltration, churning, cheese-making and fermentation, the original milk is modified to various dairy products. The complicated physical and chemical structure of milk and most dairy products often make them a difficult matrix for application of modern analytical methods. The chemical analyses made in dairying have different objectives, *e.g.*

- a) As a basis for payment to the farmer for the raw milk, *e.g.* in Sweden today according to the fat content.
- b) Test of the milk for the presence of chemical residues, *e.g.* antibiotics, heavy metals, pesticides, aflatoxins.
- c) Test of the milk for its suitability for the manufacture of certain products, *e.g.* curdforming ability for cheesemaking, content of free fatty acids, content of copper, iodine number of milk fat.

- d) Process control, *e.g.* fat content for standardization of market milk, water content in butter and milk powder.
- e) Control of ready-made products in order to assess that these meet the nutritional requirements as given by food legislation and further that products fulfil the specific palatability demands of manufacturers, *e.g.* diacetyl content in butter.

Background to the analytical investigations undertaken

(I). Metals of great technological importance in dairy products, especially butter, are Cu and Fe because even very small elevations of the normally low contents of these metals may induce lipid oxidation with severe taste deterioration as a consequence. Cu has a greater catalytical effect than Fe on the lipid oxidation. High contents of these metals in milk indicate that the milk has been contaminated, *e.g.* in pipings made of unsuitable material or that the milk originates from cows in the beginning of the lactation period.

Until ten years ago mainly colorimetric methods were used for determination of Cu and Fe in milk^{2,3}. The disadvantages of these colorimetric methods are that they are time-consuming and that the detection limits are just below the normal contents of these metals in milk. According to Dutch investigations the contents of Cu and Fe in uncontaminated milk taken directly from the teats are 20 - 40 µg/l and 100 - 250 µg/l, respectively^{2,3}. In the middle of the sixties atomic absorption spectrophotometry (AAS) began to be a common instrumental technique in analytical laboratories. Experience from our laboratory⁴, among others, has shown that the direct determination of Cu and Fe in milk is not very successful, partly because of the low contents of these metals in milk and partly because the measurements are disturbed by nonspecific absorption in the flame. The new variant for atomization of the sample for AAS-measurement, electrothermal heating, FAAS (flameless AAS)⁵, offers an improved analytical sensitivity and therefore better possibilities for a rapid and simple method for determination of the contents of Cu and Fe in milk. This application is investigated in (I).

Another metal of technological interest in milk and occurring at very low contents, 10-50 µg/l, is Mn. Mn stimulates the growth of certain aroma bacteria in starter cultures added to milk for manufacturing purposes, *e.g.* sour milk. The determination of this metal with FAAS is also studied in (I).

(II). The present debate on environmental pollution has also reached the dairy industry. Pollutants of special interest have been pesticides and heavy metals because of the great toxicity that many of these chemicals possess. Recommended daily intakes of these environmental poisons have been set by WHO (World Health Organization). Concerning the heavy metals studied in this work, Pb and Cd, the following provisional, tolerable weekly intakes have been set: Pb 3 mg and Cd 0.4-0.5 mg per capita (normal body weight).

The investigations published of the normal contents of Pb and Cd in milk have given very varying results (table 2). In table 2, the methods of analysis used are also included.

TABLE 2. Examples of reported contents of Pb and Cd in milk until 1974

Author	Coun-try	Year	Reported con- tent (µg/l)	Method of analysis
<i>Pb</i>				
Brandt & Bentz ⁶	USA	1971	1 - 10	Colorimetry
Murthy <i>et al</i> ⁷	USA	1967	49	AAS (flame)
Harding <i>et al</i> ⁸	UK	1974		AAS (flame)
Blanc <i>et al</i> ⁹	CH	1971	24	AAS (flame)
Reith <i>et al</i> ¹⁰	NL	1974	20 ± 10	AAS (flame)
Velghe <i>et al</i> ¹¹	B	1974	14 - 97	AAS (flameless)
Rüssel ¹²	D	1965	7.4 - 21.0	ASV (DC)
Stelte ¹³	D	1971	15 - 67	ASV (DC)
<i>Cd</i>				
Schroeder <i>et al</i> ¹⁴	USA	1961	1.5 - 3.6	Colorimetry
Murthy & Rhea ¹⁵	USA	1968	17 - 30	AAS (flame)
Harding <i>et al</i> ⁸	UK	1974	< 1 - 6	AAS (flame)
Kubota <i>et al</i> ¹⁶	USA	1968	6	AAS (flame)
Lener & Bibr ¹⁷	CS	1971	10 - 12	AAS (flame)
Cornell & Pallansch ¹⁸	USA	1973	0.1 - 0.4	ASV (diff.pulse)

One reason for the great variations reported in the contents of Pb and Cd in milk can of course be that the contamination of the milk during milk production differ between countries or has significantly changed in the same country during the last decade. Another reason, however, that should not be overlooked, is the reliability of the analytical methods used. The reported contents of Pb and Cd are namely often of the same order of magnitude as the detection limits of the analytical methods used. If such less sensitive methods are used, the results of analysis can be strongly affected by interferences and by contamination during the necessary sample pretreatment. The difficulties of ultratrace analysis have been thoroughly investigated by Tölg¹⁹.

The introduction of new, very sensitive methods of analysis such as DPASV (Differential Pulse Anodic Stripping Voltammetry) and FAAS has increased the possibilities for a satisfactory quantitative estimation of the contents of Pb and Cd in milk. This is investigated in (II).

(III). For good consumer acceptance the spreadability of an edible fat is an important property. Ordinary refrigerator stored butter has less good spreadability when taken directly from the cold. The solid fat content is an important parameter when considering the spreadability. The value of this parameter for an triglyceride fat like butter is dependent on the temperature and the chemical and physical properties of the component triglycerides, *e.g.* molecular weight, content of unsaturated esterified fatty acids and molecular structure. By performing temperature treatment of the cream before churning it is, however, possible to influence the solid fat content of butter within certain limits²⁰.

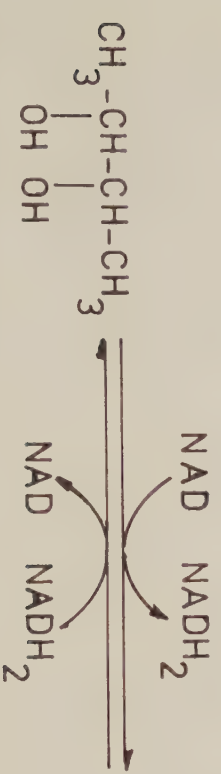
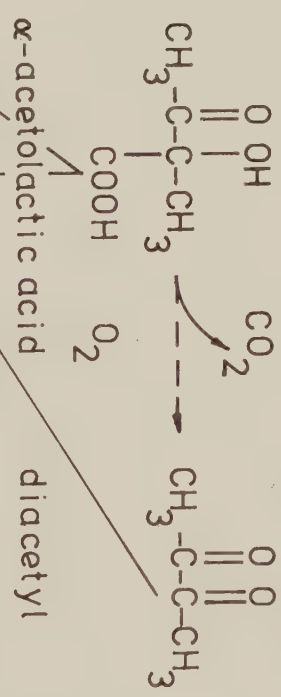
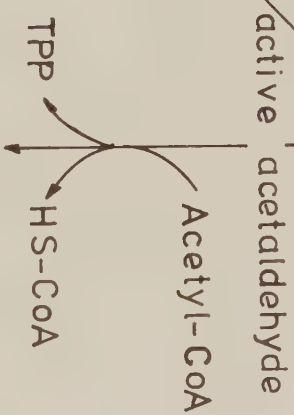
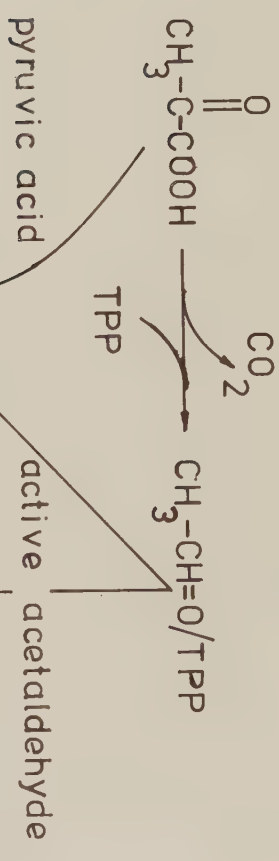
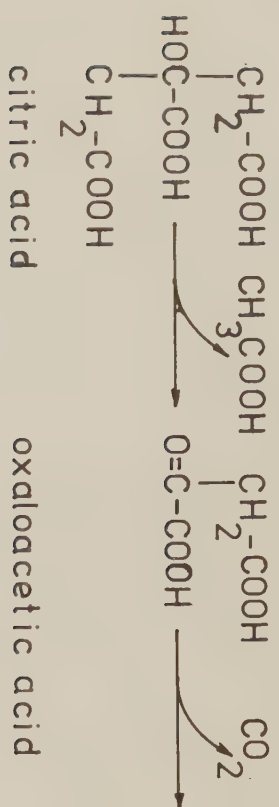
For determination of the solid fat content, dilatometry has been used for many years²¹. This method, however, is time-consuming and difficult to perform with high precision. Lately, modern methods of analysis for determination of the solid fat content, namely DSC (Differential Scanning Calorimetry) and NMR (Nuclear Magnetic Resonance) have been developed. The type of NMR used for solid fat determination has until a few years ago almost exclusively been wide-

line NMR. With the recently introduced pulse NMR, several advantages can be obtained compared to the wideline technique.

A pulse NMR spectrometer has been used for some basic studies of the crystallization of butterfat (III).

(IV and V). Fermentation is an important process in the dairy industry for manufacturing products like sour milk, butter and cheese. For fermentation starter bacteria are added to milk for production of sour milk and cheese and to cream for production of butter. A dairy starter is normally composed of both lactose fermenting bacteria (e.g. *Streptococcus* (S.) *lactis* and *S. cremoris*) for production of lactic acid and citrate fermenting bacteria (e.g. *S. Diacetylactis* and *Leuconostoc* (L.) *cremoris*) for production of aroma compounds like diacetyl and also for production of carbon dioxide. Diacetyl is an important aroma compound in products like sour milk and butter. The carbon dioxide evolved during fermentation is in most cheeses responsible for the hole formation.

Citric acid metabolism in starter culture has been studied extensively for many years. Despite all the research work done there are even today various theories as to how the fermentation of citric acid to C_4 -compounds like diacetyl, acetoin and 2,3-butylene glycol proceeds. Until about 10 years ago it was generally believed that acetoin served as a precursor for diacetyl in starter cultures. Because of the low oxidation reduction potential in the culture after some growth and the relative inertness of acetoin towards oxidation, this theory is no longer accepted. Today two main theories on the metabolic pathway for the production of diacetyl in dairy starters containing strains *S. diacetylactis* and *L. cremoris* remain. According to Pette²², de Man²³ and Seitz *et al*²⁴ diacetyl is mainly produced by a spontaneous oxidative decarboxylation of α -acetolactic acid excreted from the bacterial cells. In contrast to this non-enzymatic mechanism Speckman & Collins²⁵ assert that the formation of diacetyl is accomplished by an intracellular enzymatic condensation of acetyl-coenzyme A and activated acetaldehyde. In the metabolic scheme for the citric acid fermentation shown in figure 1 these two metabolic



butylene glycol acetoin

pathways for the formation of diacetyl are included.

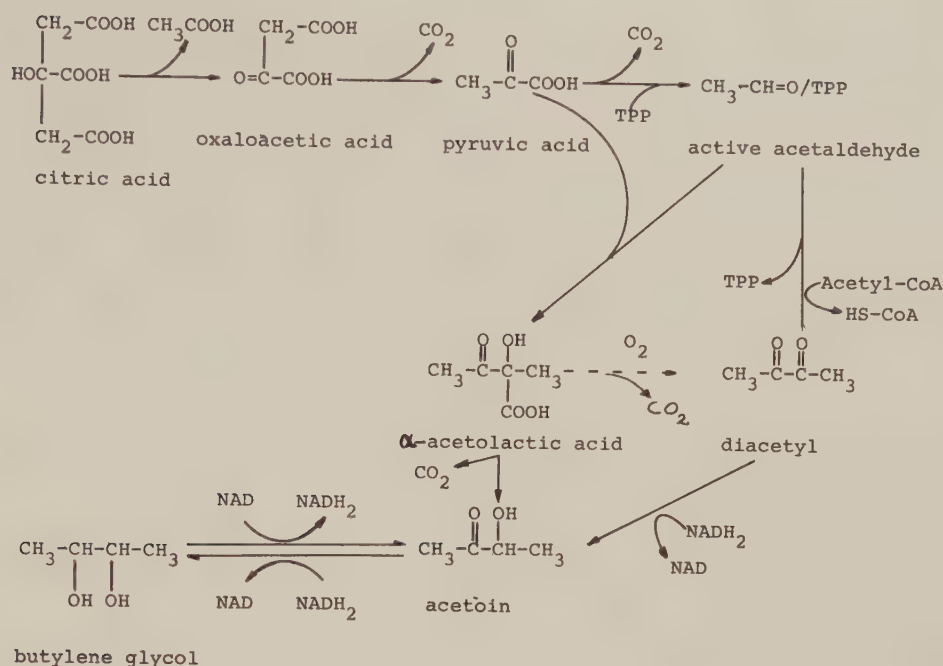


Fig. 1. Biosynthesis of diacetyl, acetoin and butylene glycol in starter cultures

Chemically the intermediary α -acetolactic acid is an unstable compound that under acidic conditions spontaneously decarboxylates to acetoin and in the presence of oxygen can also undergo oxidative decarboxylation to diacetyl²⁶.

Literature concerning α -acetolactic acid in dairy starter cultures presents varying opinions as to whether or not α -acetolactic acid is a metabolite that is accumulated and excreted from the bacterial cells. Collins & Speckman²⁶ claim that α -acetolactic acid exists only at low steady-state concentrations within the cells of the aroma bacteria, *i.e.* no excretion to the medium normally occurs. In contrast to this view-point, de Man & Pette²⁷ and Stadhouders²⁸ have presented experimental results giving evidence that the aroma bacteria studied excrete α -acetolactic acid. One reason for the existence of these diametrically opposite opinions concerning α -acetolactic acid can be traced to the analytical methods used for the determination of this unstable compound. The methods used have been based

on a forced decarboxylation reaction of α -acetolactic acid.^{29 30}

These methods have suffered from lack of selectivity and sensitivity. Due to this no systematic studies on the formation of α -acetolactic acid in starter cultures have been made. Some workers in this field have been aware of the risks that the existence of α -acetolactic acid in the starter culture might affect the accuracy of the diacetyl determination.^{23 31 32}

The aims for the experimental investigations (IV and V) made were

- a) To find an analytical procedure giving the true diacetyl content in a growing starter culture.
- b) To find a method for the determination of α -acetolactic acid.
- c) To study the formation of α -acetolactic acid, acetoin and diacetyl in some common types of aroma producing starters.
- d) To study different treatments of the culture that influence the aroma formation in lactic starters.

RESULTS AND DISCUSSION

(I) Determination of Cu, Fe and Mn in milk with FAAS

Many designs of equipment for flameless atomization in AAS have been proposed. The pioneer in this field L'vov³³ put the sample in a graphite cup which was heated by an electric arc. The atoms released by the heating were led through a graphite tube for the absorption measurement. Other devices used for flameless atomization involve applying the sample on an electrically heated carbon rod³⁴ or in a tantalum boat³⁵. The nonflame atomizer most used commercially is the graphite tube which was initially developed by Massmann³⁶. The advantage of the graphite tube is that the residence times of the atoms in the tube are relatively long which means that the absorbance signal obtained is long enough to be recorded on a common potentiometer recorder. In this work a graphite tube atomizer from Perkin Elmer AB (HGA-70) has been utilized.

The simplest way to determine Cu, Fe and Mn in milk would be a direct

injection of milk in the graphite cuvette followed by a programmed heating of the tube. This heating is divided in three steps, namely drying, ashing and atomization. Some authors^{37 38 39} have used such a simplified procedure for Cu and Fe in milk in some cases after a dilution of the milk with water. The experiences of such a methodology in this laboratory are less good due to among other things the following facts leading to poor precision

- a) High nonspecific absorbance from smoke originating from not fully pyrolysed milk. If the ashing temperature is increased in order to avoid smoke during the atomization step, losses of studied metals often occur during ashing.
- b) Deficient control of the temperature in the graphite tube because carbon residues from pyrolysed milk remain in the tube from previous injections of milk leading to poor thermal contact between the sample and the heated walls of the graphite tube. (In HGA-70 the temperature in the tube is set indirectly by the voltage applied).

In order to solve these problems a sample preparation of the milk was found almost a necessity. One pre-injection preparation which was tested is treatment of the milk with a suitable strong mineral acid. In this way the content of organic matter in the sample can be diminished (the casein precipitates) and the conditions for an efficient ashing before atomization can be improved. In order to select a proper acid for the precipitation the following mineral acids have been tested: hydrochloric, sulphuric, nitric and perchloric acid. For studying the effect of the various acids on the FAAS measurements pure standard solutions of the metals studied were used. The results of these experiments showed that the acids investigated had different effects on the peak absorbances. The acid influence varied also with the metal studied. Generally high concentration of the strongly oxidizing acids nitric and perchloric acid strongly depressed the peak absorbances especially for Mn. The least influence on the peak absorbance was obtained for hydrochloric acid but this acid had the disadvantage of giving rather high

background absorbance at the resonance line for Fe 248.5 nm. Sulphuric acid gave almost constant peak absorbances for Cu and Fe at concentrations ranging from 0.05 M to 1 M. The manganese signal decreased slightly on increased content of sulphuric acid, probably due to the fact that sulphuric acid is also oxidizing to some extent. For precipitation of milk before the FAAS analysis sulphuric acid was chosen for the determination of Cu and Fe and hydrochloric acid for Mn. A suitable concentration of acid in the milk was found to be 1 M. For calibration the method of standard additions was used.

To give an exact explanation of the interferences obtained for the various acids is very difficult. Many research groups are at present studying what is happening in the graphite tube during atomization. Sturgeon *et al*⁴⁰ have recently published a comprehensive study on this subject stating that three atomization mechanisms are operative: thermal dissociation of the analyte oxide or halide and carbon reduction of the oxide followed by atomization of the free metal. The interferences found in this study could possibly be explained in accordance with this theory meaning that the carbon reduction of the metal oxides formed is not fully accomplished at high concentrations of oxidizing acids in the sample injected.

The proposed method for Cu including a sulphuric acid precipitation of the milk sample was compared with a standard colorimetric method. The methods gave agreeing results.

The methods developed for determination of Cu, Fe and Mn have been used for a survey of the contents of these metals in Swedish market milk. The following mean values and standard deviations respectively were obtained (table 3).

TABLE 3. Contents of Cu, Fe and Mn in Swedish market milk

Metal	Mean value ($\mu\text{g/l}$)	Standard deviation ($\mu\text{g/l}$)
Cu	51	13
Fe	212	41
Mn	24	8

The contents found above of Cu, Fe and Mn in milk are in good agreement with results found abroad. Concerning the contents of Mn, milk from two dairies differed significantly from the average Mn content stated above. One of these dairies was located on the island of Gothland and showed an extremely low Mn content, namely 14 $\mu\text{g/l}$ with a standard deviation of 2.5 $\mu\text{g/l}$. The other dairy was located in the northern hilly region of Sweden. The Mn content in that milk was high, 41 $\mu\text{g/l}$ with a standard deviation of 13 $\mu\text{g/l}$.

(II) Determination of Pb and Cd in milk

To be able to determine the extremely low contents of Pb and Cd often found in milk the analytical procedures used must fulfil the following requirements

- a) High sensitivity.
- b) Freedom from interferences or when interferences exist, easy to correct for.
- c) Simple sample preparation with few transfers of the sample solution between vessels and a minimum use of reagents and other chemicals.

In order to try to meet these demands as close as possible on an analytical procedure for determination of Pb and Cd in milk the following two methods were tested, namely DPASV on a solution of a dry ashed milk sample and FAAS after an acid precipitation of the milk sample.

Background to the DPASV-technique. DC-polarography has long been used as a standard method for determination of trace metals like Pb and Cd but at relatively high concentrations because the detection limit of this method is about 10^{-5} M. By the introduction of pulse polarography the sensitivity was improved almost with a factor of 1000 because in this polarographic mode, the influence of the nonspecific capacity currents limiting the DC-technique are eliminated. In the original pulse polarography the voltage ramp was provided by voltage

pulses with linearly increasing amplitudes. In the variant of pulse polarography used in this work (II, IV), *i.e.* differential pulse voltammetry small voltage pulses are overlapped on a linearly variable DC-voltage. In the phase sensitive detector belonging to the instrument the Faraday current, if existing, is sampled differentially before and at the end of the applied voltage pulse. If this differential technique is used together with electrolytic preconcentration of the metal ions on a stationary electrode, a method (DPASV) is obtained where contents below 1 $\mu\text{g/l}$ can be measured. Concerning choice of stationary electrode, many alternatives are available today, *e.g.* the HMDE (Hanging Mercury Drop Electrode), the graphite electrode (coated with a thin mercury film) and the graphite paste electrode. In this work a HMDE from Princeton Applied Research in combination with a polarographic instrument PAR-174 from the same company were used.

The influences of some parameters affecting the sensitivity of a DPASV-analysis were investigated, namely the time of electrolysis, the stirring rate, the size of the mercury drop, the modulation amplitude and the potential scan rate. It was found that the peak heights increased with increasing time of electrolysis, stirring rate, drop size and modulation amplitude but decreased with increasing scan rate. This latter fact is in disagreement with theory and is due to the electronic time-constant of the instrument. This time-constant has been intentionally chosen high by the manufacturer in order to give the instrument a good signal-to-noise ratio.

Preparation of the milk sample before DPASV-analysis. Before a polarographic determination of Pb and Cd in milk can be performed, these metals must be isolated from the organic matrix of milk. This is best done by a wet or dry ashing of the milk. Due to the risks of contamination from the strong acids needed for a normal wet digestion of milk such a sample preparation has been considered less suitable. Instead a dry ashing procedure has been studied in some detail. Dry ashing is advantageous with respect to low risks of contamination because no concentrated acids are used and the ashing is performed

in a closed space. A number of recovery experiments (table 4) have been made at different temperatures during ashing in the muffle oven used.

TABLE 4. Recovery of Pb and Cd added to milk

Temp. °C	Recovery (%)	
	Pb	Cd
500	78 ± 3	63 ± 13
550	95 ± 7	59 ± 10
600	99 ± 11	54 ± 10

The figures in table 4 represent mean values and standard deviations of 5-10 determinations. 10 µg/l of Pb and Cd were added to the fluid milk samples used in the recovery experiments. Before dry ashing the milk samples were freeze dried. The ash was dissolved in 0.1 M HCl. The results shown in table 4 indicate that Cd is more volatile than Pb during dry ashing. This is in agreement with the results obtained by other research workers⁴¹.

Interferences on DPASV-analysis. Possible interfering elements on a polarographic determination of Pb and Cd in milk in 0.1 M HCl are mainly Ta, Sn(IV), As(III) and In, because these metals have peak potentials in the same potential range as those of the metals studied. (In the case of As(III) it is a polarographic maximum). If Ta was present, it would interfere with the Pb peak. Using the different behaviour of Pb and Ta on addition of the complexing agent EDTA, it could be shown that Ta was not present. In the same way the interference of Sn can be tested by addition of citrate or tartrate. These agents complex the Sn(IV) ions. As(III) gives rise to a polarographic maximum at contents exceeding 300 µg/l which is much higher than the maximum natural content in milk. If In was present in the dissolved milk ash it would interfere with the Cd peak but because no peak was obtained in this voltage range, it can be concluded that neither In nor Cd was

present. Moreover, In is an extremely rare metal with little industrial use.

Comparison DPASV - FAAS. In order to check the DPASV-procedure some comparative analyses were made with FAAS on injection of the supernatant after acid precipitation of the milk sample according to (I). For determination of Pb hydrochloric acid and for determination of Cd sulphuric acid were used at a concentration of 1 M. In order to compensate for the nonspecific absorption obtained on atomization a deuterium background corrector was an almost necessary accessory for these analyses because the ashing step could not be performed at temperatures high enough for a full pyrolysis to take place due to the high volatilities of Pb and Cd. The detection limits obtained with this FAAS procedure were not low enough to make absolute quantitative estimations possible. It could be established, however, that the contents of Pb and Cd in milk samples examined were below 10 µg/l and 0.5 µg/l, respectively.

Survey of contents of Pb and Cd in Swedish milk. With the DPASV-technique developed a survey of the contents of Pb and Cd in Swedish milk was carried out. In all the 98 samples analysed no detectable contents, *i.e.* < 0.2 µg/l, of Cd were found. Concerning Pb a mean value of 2.0 µg/l with a standard deviation of 0.5 µg/l was found. The highest Pb content obtained was 3.3 µg/l and the lowest 1.0 µg/l. Comparison with earlier international investigations (table 2) shows that the contents of Pb and Cd in Swedish milk are extremely low. Most investigations before 1972-73 indicated that the contents of Cd were in the interval 1.5 - 30 µg/l. The latest results, however, support our findings that the normal content of Cd in milk is below 1 µg/l.^{8,18}

Concerning the Pb contents found in Swedish milk, these are almost one tenth of earlier results published in the literature until 1975-76 (compare table 2) with exception of the results by Brandt and Bentz⁶. Recently Ellen and Tibbesma⁴² have published results from investigations in Holland that also gave low Pb contents in milk. They obtained a mean value of 4.1 µg/l. For the selection of analytical method in the work of Ellen and Tibbesma this work (II) has served as a model, *i.e.* the

method they used was based on DPASV after dry ashing of the milk. The methodology has been tested thoroughly with respect to recovery and interferences, whereby the results found in this investigation have been verified. Very recently, similarly low results of Pb in Danish milk have also been reported⁴³.

(III) Study of the crystallization behaviour of butterfat with pulse NMR

Background to the pulse NMR-technique. In this study a pulse NMR spectrometer, Bruker minispec p 20, was used. This instrument works with a proton resonance frequency of 20 MHz. For determination of the solid/liquid ratio an rf-pulse of resonance frequency is sent through the sample perpendicular to the outer magnetic field for a few microseconds whereby the spin axis of the protons can be changed 90° . By measuring the resultant magnetization signal of the protons in the plane perpendicular to the outer field, a signal is obtained, which is proportional to the number of protons in the sample. The magnetization signal obtained is not stable, however, but decays exponentially. The time constant of this process is characterized by the spin-spin relaxation time, T_2 , which is very short for protons in solids (10 μ s for solid fat protons) and long for protons in liquids (0.1 ms for liquid fat protons). It is this difference in the magnitude of T_2 that makes it possible to use pulse NMR as a quantitative method for determination of *e.g.* the solid fat content in partially crystallized fat. For this purpose the magnetization signal is electronically sampled at two different times as shown in fig. 2. The signal, 1, in fig. 2 represents the liquid signal. Due to the existence of a certain dead-time in the receiver the solid signal cannot be measured directly but must be extrapolated to zero time after the end of the pulse by aid of a calibration factor, k . The calibration factor thus needed is determined experimentally by aid of a reference oil.^{44,45} In this work (III) soybean oil has been used.

The numerical value of the calibration factor depends on temperature but also on the crystalline and chemical properties of the fat investigated. It is known that the protons of α crystals are more slowly relaxed than β' and β crystals⁴⁶. Calibration factors have been determined

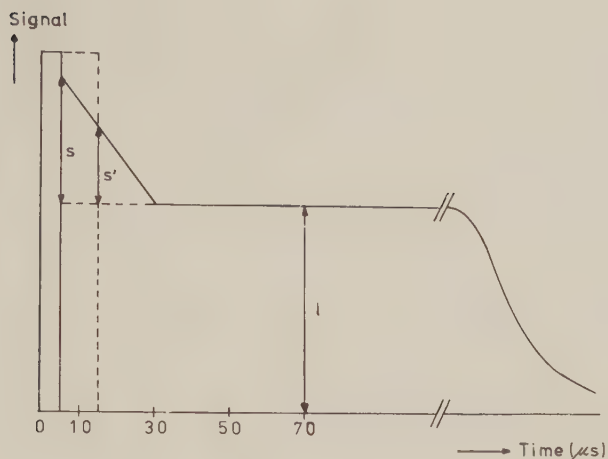


Fig. 2. The free induction decay of the magnetization signal after 90° pulse. s = solid phase, l = liquid phase

for some different margarine fats and for butterfat. At low temperatures the variations in the k-factors determined were relatively small or about 2% at 5°C but at higher temperatures the variations were greater or about 20% at 20°C . Depending on the temperature history of a fat sample before analysis, the solid fat content determined can differ markedly due to the polymorphic structures existing. If a butterfat sample is cooled rapidly from the melted state to a low temperature, *e.g.* 0°C , the formation of α -crystals is favoured. It could be shown, however, that the temperature history of the butterfat samples used for calibration was of minor importance for the k-factor value determined at least in the technologically important temperature range, $5 - 20^\circ\text{C}$.

Discussion of experimental results. The pulse NMR technique was used for some kinetic studies of the crystallization of butterfat. The results of these investigations of cooling butterfat from the melted state showed that direct cooling, and cooling at $0.5^\circ\text{C}/\text{min}$ gave about the same resultant solid fat content while cooling at $0.15^\circ\text{C}/\text{min}$ gave a lower solid fat content. A very plausible explanation to these differences can be found in the concept of mixed crystallization first postulated by Mulder⁴⁷. The outlines of the theory of mixed crystal formation are that equilibrium is not obtained during the crystalliza-

tion process, which implies that triglyceride molecules are built into the crystal structure, which from their normal melting characteristics should be in the liquid state. It is only α crystals and to some extent β' crystals that can be influenced by mixed crystallization⁴⁸. This explanation would thus mean that cooling at $0.15^{\circ}\text{C}/\text{min}$ gives a better equilibrium of crystallization than direct cooling and cooling at $0.5^{\circ}\text{C}/\text{min}$ do.

The background to the temperature treatment of the cream often made in practice before churning to butter was also investigated. The following temperature scheme, $60-7-X-14^{\circ}\text{C}$ was studied. This temperature sequence means that after melting the butterfat at 60°C , the melting fat is rapidly cooled to 7°C and is kept at this temperature for 90 minutes for equilibrium crystallization to be obtained. The next step in the temperature treatment studied was to heat the butterfat sample to an intermediate temperature, X. Temperatures in the range $16-30^{\circ}\text{C}$ were investigated and these temperatures of the fat were kept for 30 minutes, which was found appropriate for reaching equilibrium.

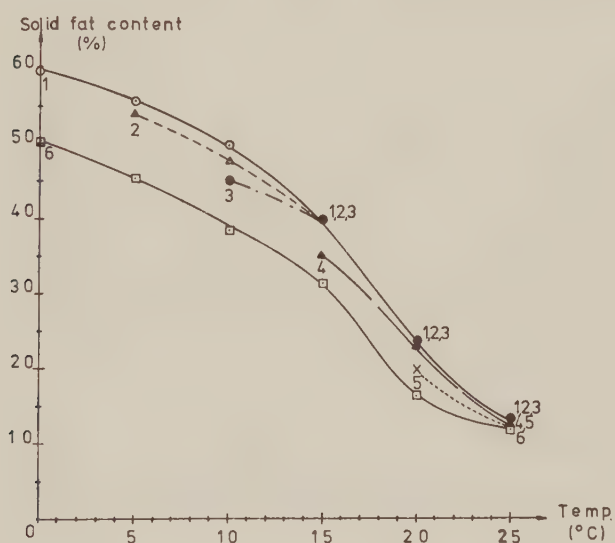


Fig. 3. Melting (1-5) and crystallization curves (6) of butterfat.

○ = (1) $60 - 0 - 5 - 10 - 15 - 20 - 25^{\circ}\text{C}$

△ = (2) $60 - 5 - 10 - 15 - 20 - 25^{\circ}\text{C}$

● = (3) $60 - 10 - 15 - 20 - 25^{\circ}\text{C}$

▲ = (4) $60 - 15 - 20 - 25^{\circ}\text{C}$

× = (5) $60 - 20 - 25^{\circ}\text{C}$

□ = (6) $60 - 25 - 20 - 15 - 10 - 5 - 0^{\circ}\text{C}$

The last step in the temperature sequence was to cool the butterfat to 14°C , representing a normal churning temperature in practice. The resulting solid fat content at this temperature was measured after 24 hours. It was found that a minimum solid fat content at 14°C was obtained after an intermediate heating to 25°C . The solid fat content thus obtained was 15% lower than after performing a temperature scheme in which the intermediate heating was omitted, *i.e.* according to the scheme $60-7-14^{\circ}\text{C}$. The explanation of this effect is almost certainly that at 25°C most of the mixed crystals previously formed at the rapid cooling to 7°C have been melted and only few mixed crystals were recreated during the subsequent cooling to 14°C . Compare fig. 3 where the space between the outer curves represent the mixed crystal region.

Evidence for the existence of mixed crystals in butterfat has recently also been found by other techniques, *e.g.* DSC and X-ray diffraction.^{48, 49}

(IV and V) Study of the citric acid fermentation in lactic starter cultures

Method for determination of diacetyl. For the determination of diacetyl in the culture distillates a new method was developed, which was based on polarography in the differential pulse mode. A stationary electrode, HMDE, was used. The diacetyl peak was obtained at -0.65 V vs. SCE . The only sample preparation needed was addition of ammonium chloride to the distillate. The method proved to be very selective for determination of diacetyl in culture distillates. This was tested by comparative analyses with gas chromatography and a standard colorimetric method. The repeatability was good, about 2.5%, expressed as the relative standard deviation.

Development of method for α -acetolactic acid and metabolic studies. When distilling a growing starter culture in N_2 and O_2 -atmospheres, respectively, a greater diacetyl content was observed in the distillate from the distillation in O_2 -atmosphere. This observation has been used as the basis for determination of α -acetolactic acid in a starter culture.

To ascertain that the compound responsible for the increase in diacetyl content really was α -acetolactic acid synthetic α -acetolactic acid was prepared and investigated on distillation under the same conditions as exist in a culture. It was found that the oxidative decarboxylation of α -acetolactic acid during distillation was influenced by primarily pH and the oxidation-reduction potential.

In an oxygen saturated skimmed medium adjusted to pH 4.5 the oxidative decarboxylation of synthetic α -acetolactic acid was about 40% on molar basis. At higher pH *e.g.* 5.5, the oxidation was reduced to about 20%. The same relative pH dependence of the oxidative decarboxylation was found for natural α -acetolactic acid on distillation of a pH-adjusted starter culture in oxygen saturated atmosphere. The length of the oxygen bubbling of the *in vitro* acidified milk medium and the starter culture samples was chosen so that equilibrium condition was reached, *i.e.* until the oxidation-reduction potential reached a plateau value which was the same for both the synthetically modified and the natural samples.

When simulating the conditions existing in a starter culture during distillation in N_2 -atmosphere, it was found that the oxidation-reduction potential in a starter culture was about 450 mV lower than in a skimmed milk sample adjusted to the same pH and with the same content of dissolved oxygen. Thus the oxidation-reduction potential in a starter culture is not only dependent on the content of dissolved oxygen but also on various other oxidation-reduction systems like $NaDH_2/NAD$. The higher oxidation-reduction potential in the skimmed milk samples adjusted to a low content of dissolved oxygen caused a partial oxidation of added α -acetolactic acid during distillation in N_2 of about 10% at pH 4.5. In contrast to this it was shown that the oxidative decarboxylation of natural α -acetolactic acid in a starter culture during nitrogen distillation was insignificant, *i.e.* the diacetyl content obtained in the N_2 -distillate represented the true diacetyl content in the starter.

The outlines of a quantitative determination of α -acetolactic acid in a starter culture thus include the following steps

a) Two identical portions of the culture are withdrawn and pH is measured

- b) One portion is distilled in a stream of nitrogen and the other in a stream of oxygen under standardized conditions.
- c) The diacetyl contents in the distillates are measured and knowing the degree of oxidative decarboxylation of synthetic α -acetolactic acid on distillation in oxygen atmosphere at the same pH a calculation of the existing content of α -acetolactic acid in the culture can be made.

In fig. 4 a typical example of the variations in the content of α -acetolactic acid in a DL-culture during growth are shown.

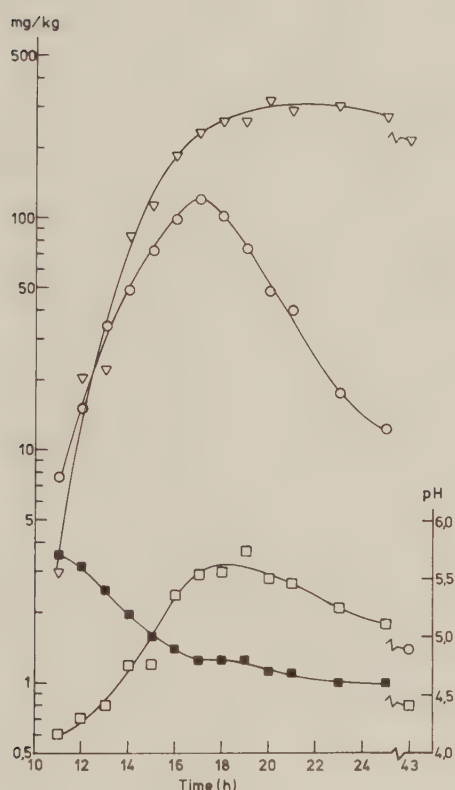


Fig. 4. Production of acetoin (▽). α -acetolactic acid (○) and diacetyl (◻) and the corresponding change in pH (■) in a growing starter culture DL-type

The corresponding contents of diacetyl and acetoin and pH are also illustrated in the same figure. The convex step of the curve for the formation of α -acetolactic acid indicates that the accumulation of this compound in the starter is controlled by two competitive chains of enzymatic reactions, namely those synthesizing and those breaking down α -acetolactic acid. At the maximum content of α -acetolactic acid

experiments have shown that the available amount of citric acid in the medium has been almost consumed. Similar metabolic curves as shown in fig. 4 have been obtained for other types of aroma cultures investigated of D- and L-type.

Results obtained in this investigation thus show that α -acetolactic acid is a metabolite excreted into the medium by the usual types of aroma bacteria which contrasts with the finding of Collins and Speckman²⁶. Concerning the metabolic formation of diacetyl it can be concluded that α -acetolactic acid is not a probable precursor to this aroma compound in a starter culture as postulated by Pette²², de Man²³ and Seitz *et al*²⁴, because of the very low oxidation-reduction potential in the culture and because of the fact that no correlation between the content of accumulated α -acetolactic acid and the content of diacetyl has been found. A pure enzymatic, intracellular formation of diacetyl is more plausible, *e.g.* according to the model proposed by Speckman and Collins²⁵.

Another important fact discovered during these aroma studies is that to determine the true diacetyl in a starter culture containing α -acetolactic acid it is almost necessary to use N₂-atmosphere during the distillation step if used in the analytical procedure. If this demand is not fulfilled, experiments have shown that diacetyl contents can be obtained that are 3-5 times higher than the real diacetyl contents.

Finally, α -acetolactic acid is a compound that can be made use of in dairy technology to increase the diacetyl content in cultured dairy products like sour milk and butter because it is easily oxidizable *e.g.* by means of an aeration procedure.

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